Metabolites of PPI-2458, a Selective, Irreversible Inhibitor of Methionine Aminopeptidase-2: Structure Determination and In Vivo Activity


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ABSTRACT

The natural product fumagillin exhibits potent antiproliferative and antiangiogenic properties. The semisynthetic analog PPI-2458, [(3R,4S,5S,6R)-5-methoxy-4-[(2R,3R)-2-methyl-3-(3-methylbut-2-enyl)oxiran-2-yl]-1-oxaspiro[2.5]octan-6-yl]-(3R,4S,5S,6R)-5-methoxy-4-[(2R,3R)-2-methyl-3-(3-methylbut-2-enyl)oxiran-2-yl]-1-oxaspiro[2.5]octan-6-yl] carbamate, demonstrates rapid inactivation of its molecular target, methionine aminopeptidase-2 (MetAP2), and good efficacy in several rodent models of cancer and inflammation with oral dosing despite low apparent oral bioavailability. To probe the basis of its in vivo efficacy, the metabolism of PPI-2458 was studied in detail. Reaction phenotyping identified CYP3A4/5 as the major source of metabolism in humans. Six metabolites were isolated from liver microsomes and characterized by mass spectrometry and nuclear resonance spectroscopy, and their structures were confirmed by chemical synthesis. The synthetic metabolites showed correlated inhibition of MetAP2 enzymatic activity and vascular endothelial cell growth. In an ex vivo experiment, MetAP2 inhibition in white blood cells, thymus, and lymph nodes in rats after single dosing with PPI-2458 and the isolated metabolites was found to correlate with the in vitro activity of the individual species. In a phase 1 clinical study, PPI-2458 was administered to patients with non-Hodgkin lymphoma. At 15 mg administered orally every other day, MetAP2 in whole blood was 80% inactivated for up to 48 hours, although the exposure of the parent compound was only ~10% that of the summed cytochrome P450 metabolites. Taken together, the data confirm the participation of active metabolites in the in vivo efficacy of PPI-2458. The structures define a metabolic pathway for PPI-2458 that is distinct from that of TNP-470, [(3R,4S,5S,6R)-5-methoxy-4-[(2R,3R)-2-methyl-3-(3-methylbut-2-enyl)oxiran-2-yl]-1-oxaspiro[2.5]octan-6-yl] N-(2-chloroacetyl)carbamate. The high level of MetAP2 inhibition achieved in vivo supports the value of fumagillin-derived therapeutics for angiogenic diseases.

Introduction

The fungal metabolite fumagillin (Fig. 1A) has been associated with potent cytostatic activity on endothelial cells that led to demonstrations of an antiangiogenic effect in vivo, thereby offering a novel mechanism for treatment of cancer and other diseases dependent on blood vessel formation (Ingber et al., 1990; Folkman, 2007; Ribatti, 2009; Szekanecz et al., 2009; Mauriz et al., 2010; Gamba-Sanchez, 2012; Yin et al., 2012). An early medicinal chemistry campaign seeking to develop fumagillin analogs as therapeutics culminated in the discovery of the analog TNP-470, [(3R,4S,5S,6R)-5-methoxy-4-[(2R,3R)-2-methyl-3-(3-methylbut-2-enyl)oxiran-2-yl]-1-oxaspiro[2.5]octan-6-yl] N-(2-chloroacetyl)carbamate (Marui et al., 1992). This compound, which potently inhibited tumor progression in a number of animal models, entered clinical practice in 1992. Despite encouraging preliminary findings against
several cancers (Folkman, 1998; Bernier et al., 2005; Lefkove et al., 2007), further progress of TNP-470 in the clinic setting was hampered by dose-limiting central nervous system (CNS) toxicity, lack of oral bioavailability, and rapid clearance. The chloroacetamide functionality was found to be highly labile, degrading to an unsubstituted carbamate that was processed further by epoxide hydrolase (Placidi et al., 1995).

The molecular target of fumagillin has been identified as the enzyme methionine aminopeptidase-2 (MetAP2), whose cellular role is to cleave the N-terminal methionine residue from newly synthesized polypeptide chains (Griffith et al., 1997; Sin et al., 1997). The crystal structure of the fumagillin-enzyme complex subsequently revealed a covalent binding interaction, wherein the active site His-231 was irreversibly modified by reaction with the fumagillin spiroepoxide (Liu et al., 1998; Fig. 1B). In this complex, the hexenyl chain of fumagillin mimics the Met thioether functionality, whereas the polyolefinic carboxylate extends out of the active site. PPI-2458, [(3R,4S,5S,6R)-5-methoxy-4-[(2R,3R)-2-methyl-3-(3-methylbut-2-enyl)oxiran-2-yl]-1-oxaspiro[2.5]octan-6-yl]-N-[(2R)-1-amino-3-methyl-1-oxobutan-2-yl]carbamate, differs from fumagillin in that the polyolefinic chain is replaced by a carbamoyl-linked D-valinamide moiety (Olson et al., 2003; Arico-Muendel et al., 2009). PPI-2458 has demonstrated efficacy in rodent models for non-Hodgkin lymphoma (Cooper et al., 2006), melanoma (Hannig et al., 2006), and arthritis (Bernier et al., 2004; Bainbridge et al., 2007; Hannig et al., 2007; Lazarus et al., 2008; Brahn et al., 2009; Ashraf et al., 2010; Ashraf et al., 2011). In preclinical studies, PPI-2458 was approximately equipotent in several in vivo efficacy models by both oral and parenteral routes of administration, despite an apparently modest oral bioavailability (F% = 6) (Arico-Muendel et al., 2009). To resolve this issue, we initiated efforts to identify the metabolites of PPI-2458 and to characterize their MetAP-2 inhibitory activity. Here, we report the structures of the six predominant metabolites formed by exposure of PPI-2458 to liver microsomal preparations from multiple species, and characterize their formation by cytochrome P450 (P450). All metabolites retained significant, though varying, MetAP2 inhibitory and antiproliferative activities. We also describe the in vivo inhibition of MetAP2 in blood and several tissues of rats found by dosing with the individually synthesized metabolites and comparing with PPI-2458, using an ex vivo enzyme-linked immunosorbent assay (ELISA) assay to determine free MetAP2 levels (Bernier et al., 2004). Lastly we report on pharmacokinetic (PK) and pharmacodynamic (PD) results from two cancer patients treated with PPI-2458 as part of a phase 1 clinical trial. This study revealed highly efficient inhibition of circulating MetAP2 in conjunction with rapid metabolite formation, confirming the importance of active metabolites in the efficacy of PPI-2458.

### Materials and Methods

#### General

Commercially available reagents and solvents were used as purchased without further purification. Midazolam, nifedipine, 1,2-hydroxymidazolam, oxidized nifedipine, and ketoconazole were from Ultrafine (Manchester, United Kingdom). PPI-2458 was prepared from fumagillin (Arico-Muendel et al., 2009). NADPH (Sigma-Aldrich, St. Louis, MO) was prepared as a 10 or 20 mM stock solution in H2O. Potassium phosphate buffer (KPO4) was prepared as a 100 mM stock solution, and the pH was adjusted to 7.4. Microsomes (human, cynomolgus monkey, beagle dog, Sprague-Dawley rat, and CD1 mouse) and S9 liver fractions (male Sprague-Dawley rat) were obtained from In Vitro Technologies (Baltimore, MD) or XenoTech (Lenexa, KS). Equal volumes of microsomes derived from males and females were combined for each species. Supersomes containing recombinant human CYP3A4 or CYP3A5 plus P450 reductase were purchased, along with the insect expression system preparations from multiple species, and characterize their formation by cytochrome P450 (P450). All metabolites retained significant, though varying, MetAP2 inhibitory and antiproliferative activities. We also describe the in vivo inhibition of MetAP2 in blood and several tissues of rats found by dosing with the individually synthesized metabolites and comparing with PPI-2458, using an ex vivo enzyme-linked immunosorbent assay (ELISA) assay to determine free MetAP2 levels (Bernier et al., 2004). Lastly we report on pharmacokinetic (PK) and pharmacodynamic (PD) results from two cancer patients treated with PPI-2458 as part of a phase 1 clinical trial. This study revealed highly efficient inhibition of circulating MetAP2 in conjunction with rapid metabolite formation, confirming the importance of active metabolites in the efficacy of PPI-2458.

#### Abbreviations

- **AUC**: area under the curve
- **BLQ**: below limit of quantitation
- **CL/F**: initial dose bioavailability adjusted clearance
- **ELISA**: enzyme-linked immunosorbent assay
- **ESI+**: positive electrospray ionization
- **HPLC**: high-pressure liquid chromatography
- **HUVEC**: human umbilical vascular endothelial cell
- **KPO4**: potassium phosphate buffer
- **KTZ**: ketoconazole
- **LC–MS**: liquid chromatography–mass spectrometry
- **LC–MS/MS**: liquid chromatography–tandem mass spectrometry
- **LLOQ**: lower limit of quantitation
- **MetAP2**: methionine aminopeptidase-2
- **MRM**: multiple reaction monitoring
- **PB**: pharmacokinetic
- **P450**: cytochrome P450
- **PBS**: phosphate-buffered saline
- **PD**: pharmacodynamic
- **PPI-2458**: [3R,4S,5S,6R]-5-methoxy-4-[(2R,3R)-2-methyl-3-(3-methylbut-2-enyl)oxiran-2-yl]-1-oxaspiro[2.5]octan-6-yl]-N-[(2R)-1-amino-3-methyl-1-oxobutan-2-yl]carbamate
- **PPI-4338**: [3R,4S,5S,6R]-4-[(2R,3R)-3-(3-hydroxy-3-methylbutyl)-2-methyloxiran-2-yl]-5-methoxy-1-oxaspiro[2.5]octan-6-yl]-N-[(2R)-1-amino-3-methyl-1-oxobutan-2-yl]carbamate
- **RT**: retention time
- **Tmax**: time of maximum plasma concentration
- **TNP-470**: [3R,4S,5S,6R]-5-methoxy-4-[(2R,3R)-2-methyl-3-(3-methylbut-2-enyl)oxiran-2-yl]-1-oxaspiro[2.5]octan-6-yl]-N-[(2R)-1-amino-3-methyl-1-oxobutan-2-yl]carbamate
- **Vss/F**: steady-state volume of distribution
guidelines, in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health.

**LC-MS Analysis of In Vitro Metabolism of PPI-2458 by Liver Microsomes**

**Sample Preparation.** Three incubations were prepared for each microsome evaluation: (A) time zero control, (B) no-NADPH control, and (C) complete incubation. A stock solution was prepared for each microsome evaluation by combining 891 μl of 100 mM KPO4, 25 μl of male/female pooled microsomes, and 4 μl of 5 mM PPI-2458. Stock solution (230 μl) was then aliquoted into each of three 1.5 ml microfuge tubes. The tubes were preincubated for 5 minutes at 37°C. Time zero control samples were then quenched with 500 μl of CH3CN followed by the addition of 20 μl of 10 mM NADPH. No-NADPH control samples were treated with 20 μl of H2O while complete incubation samples received 20 μl of 10 mM NADPH; the samples were then incubated at 37°C for 20 minutes with constant shaking at 600 rpm, followed by quenching with 500 μl of CH3CN. The samples were then vortexed briefly and centrifuged at 14,000 rpm, and the supernatants were evaporated under nitrogen for 20 minutes at 45°C. The samples were then taken up in 100 μl of H2O, recentrifuged, and the supernatants were transferred to autosampler vials for LC-MS analysis. An additional control incubation was performed substituting water in the place of PPI-2458. Incubations with male Sprague-Dawley rat liver S9 were performed as already described except that the initial stock solution was prepared by combining 816 μl of 100 mM KPO4, 100 μl of male rat liver S9, and 4 μl of 5 mM PPI-2458.

**LC-MS for Full Scan Analysis of Microsome Incubations.** Chromatograms were acquired on an Applied Biosystems API 4000 (Framingham, MA) triple quadrupole mass spectrometer operating in positive electrospray ionization (ESI+) full scan mode with acquisition from 300 to 800 amu. LC was performed as follows: column, 2.1 × 150 mm Symmetry C18; mobile phases: A = 85:5:10 H2O/CH3CN/25 mM NH4OAc (aq); B = 90:10 CH3CN/25 mM NH4OAc (aq), flow rate of 200 μl/min, and column temperature 35°C. The injection volume was 20 μl, and the gradient 10% B for 5 minutes then 10% → 50% B in 30 minutes.

**Inhibition of PPI-2458 Metabolism by Ketoconazole**

Coincubations of PPI-2458 with KTZ (ketoconazole) were performed using male human liver microsomes. A stock was prepared by combining 3.161 ml of 5 mM PPI-2458, and divided among 12 microfuge tubes as 217.5 μl aliquots. Duplicate tubes were then treated with 12.5 μl of ketoconazole (KTZ) stock solutions (10 mM in ethanol) diluted to 2% ethanol-water at the following KTZ concentrations: 200 μM, 20 μM, 2 μM, 200 nM, 0 nM (vehicle), and 0 μM (vehicle, no NADPH). All the tubes were preincubated for 5 minutes at 37°C before addition of 20 μl of 20 mM NADPH (or 20 μl of water in the case of the no NADPH control). The final KTZ concentrations were 10 μM, 1 μM, 100 nM, 10 nM, or 0 nM in a volume of 250 μl; the final concentration of PPI-2458 was 150 μM. The samples were then incubated for 20 minutes at 37°C with shaking at 600 rpm. The incubations were quenched with 500 μl of CH3CN followed by 100 μl of internal standard solution. The samples were vortexed and then centrifuged at 14,000 rpm for 10 minutes. A 10 μl aliquot of each supernatant was transferred to an autosampler vial containing 190 ml of H2O, and the samples were analyzed by multiple reaction monitoring (MRM) by LC-MS. Area ratios of the parent compound to the internal standard were averaged between the duplicates.

**Liquid Chromatography--Tandem Mass Spectrometry Analysis of KTZ Inhibition Samples.**

Chromatograms were acquired on an Applied Biosystems API 3000 triple quadrupole mass spectrometer operating in ESI+ mode with monitoring at 442.4, 450.4, 458.4, and 474.4 amu for Q1 mass, and 375.2, 383.3, 391.2, and 231.2 amu for Q3 mass for PPI-2458, PPI-2458-day8 (internal standard), +16 metabolite, and +32 metabolite, respectively. LC was performed as follows: column, 2.1 × 30 mm C8; mobile phases: A = 85:5:10 H2O/CH3CN/25 mM NH4OAc (aq); B = 90:10 CH3CN/25 mM NH4OAc (aq). The flow rate was 400 μl/min, column temperature was 35°C; injection volume was 20 μl; and gradient was 20% → 90% B in 3.0 minutes.

**NMR Analysis of In Vitro Metabolism of PPI-2458 by Liver Microsomes**

**Large-Scale Sample Preparation.** A general procedure for microsomal incubations of PPI-2458 on a 5-mg scale was developed as follows. PPI-2458 and mouse liver microsomes were combined in 100 mM KPO4 buffer (pH 7.4) and preincubated at 37°C for 3 minutes with constant orbital shaking at 600 rpm. After the preincubation period, NADPH was added to initiate the reaction. Final concentrations after addition of NADPH were 80 μg/ml PPI-2458, 40 μl/ml microsomes, and 1.3 mg/ml NADPH. Incubations proceeded at 37°C with continued shaking for a total of 60 minutes. An additional aliquot of PPI-2458 (~80 μg/ml) was added 15 minutes into the incubation, and an additional aliquot of NADPH (~0.66 mg/ml) was added 20 minutes into the incubation in an effort to maximize the yield of metabolites. After 60 minutes, the preparations were centrifuged to remove the microsomes, and the aqueous layer was extracted with dichloromethane (3 × 60 ml). The organic extract was concentrated by rotary evaporation, and the resulting residue was dissolved in ethanol. Individual metabolites were purified from the metabolite mixture obtained from the microsomal incubations by use of analytical high-pressure liquid chromatographic (HPLC) and fraction collection. Preparative LC was performed as follows: column, 4.6 × 150 mm Waters Symmetry C18 column (Waters, Milford, MA); mobile phases: A = H2O, B = CH3CN; flow rate, 700 μl/min; column temperature, ambient; gradient, 20% B for 10 minutes then 20% → 40% B in 30 minutes. As necessary, fractions were analyzed by LC-MS to confirm identity and purity. Fractions were pooled to obtain individual metabolites at >90% homogeneity, and were lyophilized before NMR studies to yield 100–500 μg of each metabolite.

**NMR Spectroscopy.** Samples of metabolites purified from microsomal preparations were dissolved in 20% CH3CN in D2O and lyophilized to reduce the NMR signal due to residual water. The lyophilisates were then dissolved in 200 μl of CD3OD (99.96% atom D; Cambridge Isotope Laboratories, Andover, MA) and transferred to CD3OD susceptibility-matched microcell NMR tubes (part MMS-005V; Shigemi, Inc., Allison Park, PA). Chemically synthesized metabolites were dissolved in the CD3OD (~10 mg in 1 ml) and tested in standard 5 mm NMR tubes. NMR was performed on a Varian Unity Innova 600 MHz spectrometer (Varian, Inc., Palo Alto, CA) operated by the Chemistry Department at Brandeis University, and on a Varian MercuryPlus 400 MHz spectrometer at Praccis Pharmaceuticals, Inc. One-dimensional 1H spectra were recorded at 25°C on the 600 MHz spectrometer, and at ambient temperature on the 400 MHz spectrometer. One-dimensional 13C and gradient-enhanced two-dimensional double-quantum filtered correlation (DQ-COSY), total correlation spectroscopy (TOCSY), and rotating frame Overhauser effect spectroscopy (ROESY) were recorded using standard acquisition and processing parameters.

**Chemical Synthesis of Metabolites.**

To confirm structures as deduced by LC-MS and NMR analyses and to provide materials in sufficient quantities for in vivo testing, the six major metabolites (M1–M6) were chemically synthesized (Supplemental Data protocol, references, and Supplemental Fig. 1).

**Determination of P450 Enzymes Involved in the Metabolism of PPI-2458**

The study employed a reaction phenotyping kit from Xenotech (part no. H0500, version 6). The NADPH regenerating system solution was prepared from 15 mg of NADP+, 195 μl of 1 M glucose-6-phosphate in 100 mM phosphate buffer, 60 μl of 325 U/ml glucose-6-phosphate dehydrogenase in 100 mM phosphate buffer, 60 μl of 1 M MgCl2, and 7.5 ml of phosphate buffer, pH 7.4. PPI-2458 (2.5 ml of 10 μg/ml aqueous solution) was diluted into 12.0 ml of phosphate buffer, and 725 μl was aliquotted into each of 17 silanized Empendorf tubes. Human liver microsomes (20 mg/ml) from the phenotyping kit were rapidly thawed from frozen 40 μl aliquots stored at −80°C, and 25 μl of each was added to a separate aliquot of PPI-2458/buffer mixture. Each PPI-2458/microsome sample was then divided into four aliquots of 150 μl each (one T0 sample plus triplicate reaction runs). Samples were equilibrated at 37°C for 5 minutes, and T5 samples were then immediately quenched with 500 μl of CH3CN, then treated with NADPH regenerating solution. The other samples were treated with 100 μl of NADPH regenerating solution, mixed, and quenched with CH3CN after 4 minutes of incubation. Internal standard solution (2 μl of deuterated PPI-2458, 1 mg/ml in ethanol)
was then added to each sample. Samples were then each diluted with 250 μl of water, vortexed and centrifuged at 14,000 rpm for 10 minutes, and 80 μl of supernatant were then further diluted with 120 μl of water and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) MRM for the parent compound, +16, and +32 metabolites. Chromatograms were acquired on an Applied Biosystems API 4000 triple quadrupole mass spectrometer operating in ESI+ mode with MRM Q1 and Q3 transition mass (amu) as follows: PPI-2458, 442.4 and 375.2; PPI-2458-day8 (internal standard), 450.4 and 383.3; +16 metabolites, 458.4 and 391.2; and +32 metabolites, 474.4 and 231.2. LC was performed as follows: column, 2.1 × 150 mm Symmetry C18; mobile phases: A = 85:5:10 H2O/CH3CN/25 mM NH4OAc (aq), B = 95:5 CH3CN/25 mM NH4OAc (aq); and flow rate 200 μl/min, column temperature 35°C, injection volume 20 μl, and gradient 10% B for 5 minutes then 10% → 50% B in 30 minutes.

Inhibition of P450s in Microsomal Preparations and Kinetics of PPI-2458 by Recombinant CYP3A4 and CYP3A5

**Enzyme Assays.** Microsomal incubations were performed in silanized microcentrifuge tubes at 37°C in a Thermomixer (Eppendorf, Hauppage, NY) with constant mixing at 400 rpm. Microsomes were initially diluted to 5 times (5×) the final concentrations in cold phosphate buffer and kept on ice along with a 5× NADPH-generating solution. Substrate and inhibitor stock solutions were diluted into water, and final incubation mixtures contained 0.45% acetonitrile or less. Aliquots of the 5× NADPH-generating solution were warmed in the mixer for at least 5 minutes before use. Microsomes were preincubated for 5 or 10 minutes with the test articles by adding 40 μl of the cold 5× microsome suspension to 120 μl of warmed substrate ± inhibitor solution in the mixer. Incubations were initiated by adding 40 μl of 5× NADPH-generating solution. The final incubation volume was 200 μl and contained 1.0 mM NADP+, 5.0 mM glucose-6-phosphate, 1.0 U/ml glucose-6-phosphate dehydrogenase, 3.0 mM magnesium chloride, and 50 mM phosphate buffer. The reactions were halted by protein precipitation, transferring 100 μl of the mixtures to 600 μl of ice-cold 80% acetonitrile containing PPI-2458 D-Deu analog as internal standard. Matrix standards were prepared by stepwise additions of product solutions and each incubation mixture component to cold internal standard solution. Samples and standards were stored at −20°C and then centrifuged at 14,000 rpm for 10 minutes. The supernatants were decanted into fresh silanized microcentrifuge tubes and either diluted 1:20 into 10% CH3CN or dried under nitrogen in a TurboVap (Zymark, Hopkinton, MA) set into fresh silanized microcentrifuge tubes and either diluted 1:20 into 10% CH3CN or dried under nitrogen in a TurboVap (Zymark, Hopkinton, MA) set to 35°C and reconstituted in 100 μl of 10% CH3CN for quantitation by LC-MS/MS.

Initial rate conditions and estimates of $K_m$ were established by incubating 1.5–15.0 μM midazolam, 7.5–50.0 μM nifedipine, or 15–55 μM testosterone for 1–8 minutes in the presence of 0.1 or 0.2 mg/ml human liver microsomes. Inhibition of 1′-hydroxymidazolam, oxidized nifedipine, and 6β-hydroxytestosterone formation by 0.02–0.5 μM ketoconazole or 25–400 μM PPI-2458, in the presence of 0.05 or 0.2 mg/ml human liver microsomes, was evaluated over a range of 1.5–90 μM midazolam, 20–100 μM nifedipine, or 25–150 μM testosterone for 1–4 minutes. The kinetics of formation for the four major single oxidation metabolites of PPI-2458 by CYP3A4 and CYP3A5 superposers were evaluated over a range of 25–800 μM in the presence of 40 nM P450, 80 nM P450, or 40 nM P450 plus 40 nM equivalent insect cell protein control for 3–18 minutes.

**LC-MS/MS Analysis.** Samples (5–25 μl injections) were analyzed by LC-MS/MS using an API 3000 triple quadrupole mass spectrometer in ESI+ ionization mode with MRM Q1 and Q3 transition mass (amu) as follows: midazolam, 326.2 and 291.1; 1′-hydroxymidazolam, 342.2 and 203.2; nifedipine, 347.2 and 254.3; oxidized nifedipine, 345.1 and 284.3; testosterone, 456.3 and 389.2; 6β-hydroxytestosterone, 305.2 and 269.2; +16 metabolites, 458.4 and 391.4; +32 metabolites, 474.3 and 231.1; PPI-2458, 442.3 and 375.2; and PPI-2458-Leu (IS), 456.3 and 389.2. HPLC mobile phases were as follows: A = 85:5:10 H2O/CH3CN/25 mM NH4OAc (aq), pH 3.5; B = 90:10 CH3CN/25 mM NH4OAc (aq), pH 3.5. Probe substrate metabolites were monitored using a Clipped C8 column (5 μm; 2.1 × 30 mm; Higgins Analytical, Mountain View, CA), with a linear gradient of 20% → 90% B over 3 minutes at a flow rate of 0.4 ml/min. PPI-2458 metabolites were monitored using a Symmetry C18 column (3.5 μm; 2.1 × 30 mm; Waters), with a gradient of 15% → 22%B over 3 minutes and 22% → 90% B over 3 minutes at a flow rate of 0.5 ml/min. Column temperatures were maintained at 35°C.

**Data Analysis.** Kinetic parameters were determined by nonlinear regression analysis of the data using Excel Solver (v. 2002; Microsoft, Redmond, WA). PPI-2458 metabolite production data from recombinant CYP3A4 and CYP3A5 incubations were fit to the Michaelis-Menten model (eq. 1). CYP3A substrate inhibition data from human liver microsome incubations with and without inhibitor were simultaneously fit to the Michaelis-Menten model of competitive inhibition (eq. 2), several models of atypical kinetics (Galetin et al., 2003) for single-binding-site positive cooperative inhibition or negative cooperativity with partial inhibition (eqs. 3 and 4), and multiple-binding-site substrate inhibition or heterotropic inhibition (not shown).

\[
v = \frac{V_{\text{max}}[S]}{K_m + [S]},
\]

\[
v = \frac{V_{\text{max}}[S]}{K_m (1 + \frac{[I]}{K_i})} + [S],
\]

\[
v = \frac{V_{\text{max}}[S]}{K_m (1 + \frac{[I]}{K_i})} + [S] + \frac{\gamma[S][I]}{\delta K_{i} K_r},
\]

\[
v = \frac{V_{\text{max}}[S]}{K_m (1 + \frac{[I]}{K_i})} + [S] + \frac{\gamma[S][I]}{\delta K_{i} K_r}.
\]

where $\alpha$, $\gamma$, and $\delta$ correspond to interaction factors associated with inhibitor-binding affinity, efficiency of product formation, and substrate-binding affinity, respectively.

The choice of best-fitting model was based on a minimized sum of the squared residuals (SSR), the average SSR from 100 Monte Carlo simulations, and visual inspection of the fit and simplicity of the model. A weighting factor of 1/1 was used in the final inhibition kinetics analysis.

**MetAP2 and Human Umbilical Vascular Endothelial Cell Activity**

Metabolites were tested against hMetAP2 and human umbilical vascular endothelial cells (HUVEC), as described previously elsewhere (Arico-Muendel et al., 2009). For the HUVEC assay, metabolites were tested at final concentrations ranging from 0.02 pM to 2 μM.

**In Vivo MetAP2 Inhibition by PPI-2458 and Metabolites in Rodents**

**Compound Formulation.** PPI-2458 and metabolites were formulated in 11% hydroxypropyl-β-cyclodextrin (HPCD) in water for injection at 0.03, 0.06, and 0.3 mg/ml.

**Species.** Male Sprague-Dawley rats (200–225 g), purchased from Charles River Laboratories and housed two per cage, were provided with water and chow available ad libitum, and allowed to acclimate 5 days before the study start.

**Dose.** Animals administered the test articles orally were dosed using 18G feeding needles and 3 ml syringes. Animals administered the test article intravenously were dosed using 25G butterfly infusion sets and 5 ml syringes. Each study group contained three animals. PPI-2458 and M1, M2, M3, and M4 were each dosed intravenously at 0.3 mg/kg, and orally at 0.3 and 3.0 mg/kg.

**Sample Collection.** Blood (approximately 500 μl) was collected from the jugular vein of each conscious rat −4 hours after compound administration. Approximately 24 hours after compound administration, the animals were anesthetized using isoflurane inhalant anesthesia, and blood was collected via cardiac puncture before sacrificing the animals with carbon dioxide. After euthanasia, the lungs, liver, thymus, and pooled lymph nodes (various, not including mesenteric) were collected from each animal.

**Sample Preparation.** Immediately after the collection at 4 hours, blood was placed into microtainers containing EDTA. Samples were placed briefly on a tilt table, then centrifuged at 6000g for 8 minutes in an Eppendorf centrifuge (Brinkmann Inc., Westbury NY). Plasma supernatant was pipetted into 1.2 ml...
sianized Eppendorf tubes (Corning, Corning NY) and stored at \(-80\)°C until analysis. Immediately after the collection at 24 hours, approximately 500 µl of each sample was placed into microtainers containing EDTA, then processed to plasma as at 4 hours. The remaining blood samples, \(-5\) ml/rat, were placed into 5 ml tubes containing EDTA, then pooled by study group into 50 ml conical tubes and placed on ice until analyzed for MetAP2 content. Organs were immediately snap frozen with liquid nitrogen and stored at \(-80\)°C until analysis.

### Plasma Sample Analysis

Plasma samples were thawed to room temperature to determine drug levels of PPI-2458 and metabolites. For each sample, 200 µl was transferred to a microcentrifuge tube to which 50 µl of a 50 ng/ml solution of PPI-2458-d8 was added as an internal standard. Standard curves were prepared for each analyte in blank plasma over a compound range from 0.1–100 ng/ml. Plasma protein was precipitated by adding 500 µl of acetonitrile and vortexing. The tubes were then centrifuged at 14,000 rpm for 10 minutes at room temperature. The resulting supernatants were transferred to clean microcentrifuge tubes and dried under nitrogen for 30 minutes at 80°C. The samples were then reconstituted with water to \(-100\) µl followed by vortexing and centrifugation. The resulting supernatants were transferred to autosampler vials, and 40 µl were injected onto a C8 HPLC column and separated by a 4.5-minute gradient elution. LC-MS/MS analysis was achieved with an API 4000 triple quadrupole mass spectrometer using multiple reaction monitoring data acquisition including transitions for PPI-2458 (442→375), PPI-2458-d8 internal standard (450→383), and a single transition appropriate to metabolites (458→391 for M1-M4). Quantitation was performed using Analyst 2.1 software (Perkin-Elmer Sciex, Norwalk, CT).

### Determination of In Vivo MetAP2 Inhibition

Whole blood samples were lysed for 30 minutes at 2–8°C (50 mM TrisHCl, pH 7.4; 1% NP-40 (Calbiochem, San Diego, CA), 0.25% Na deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM Na2VO4, and 1 mM NaF), vortexed, centrifuged 10 minutes at 13–14 K rpm, decanted, and the supernatants frozen and stored at \(-80\)°C. Tissue samples (\(-0.2\) g each) were homogenized in disposable tissue grinders (Corning) in \(-5\) volumes of phosphate-buffered saline (PBS) and complete protease inhibitor. Samples were then treated with 120 ml of 10% NP-40 in PBS, centrifuged, and the supernatants frozen and stored at \(-80\)°C. Levels of free MetAP2 were quantitated as previously described elsewhere (Bernier et al., 2004). Briefly, samples were treated with a biotinylated analog of PPI-2458 to covalently label all noninhibited MetAP2 in the sample. The complex of MetAP2 with inhibitor-biotin conjugate was then captured on streptavidin plates (Pierce Biotechnology, Rockford, IL) and detected in ELISA format using an anti-MetAP2 antibody.

### PK/PD of PPI-2458 in Humans

#### Study Design, Subjects, and Dosing

This was an open label, phase I safety/tolerance study of PPI-2458 in patients with non-Hodgkin lymphoma. The study was conducted at Dana-Farber Cancer Institute, Boston, MA. Written informed consent was obtained from each patient after explanation of the aims, methods, anticipated benefits, and potential hazards of the study. Informed consent was obtained before any study-related procedures were performed. The study was conducted in accordance with generally accepted standards for the protection of subject safety and welfare and in compliance with the informed consent regulations of 21 CFR §50, institutional review board/ethics committee regulations of 21 CFR §56, and the principles of the Declaration of Helsinki and its amendments.

Two patients were enrolled in the initial cohort of the study. Patient 1 was a 70-year-old Caucasian female who weighed 61.9 kg; her diagnosis was diffuse large B cell lymphoma. Patient 2 was a 66-year-old Caucasian male who weighed 104.5 kg; his diagnosis was follicular lymphoma. The planned dosing schedule was for 15 mg PPI-2458 administered by mouth (capsules) every other day for 21 days (11 doses total), followed by a 7-day recovery period. Patient 1 completed 21 days of dosing and was withdrawn from the study due to disease progression. Due to a preclinical neurotoxicity finding, patient 2 was withdrawn from the study on day 5 of dosing, and the study was terminated.

#### Sample Collection and Analysis

Samples for pharmacokinetic analyses were collected before the initial dose and at the following sampling times after the first dose: 15 minutes, 30 minutes, 1 hour, and 2, 3, 4, 6, 8, 24, and 48 hours. Pharmacokinetic samples were also scheduled for collection at predose and 4 hours after the dose on day 15. Processing of pharmacokinetic samples was performed at Charles River Laboratories (Worcester, MA). Specifically, an LC-MS/MS method was developed to quantitate levels of PPI-2458 and the four +16 metabolites in blood plasma after precipitation of protein, evaporation, and sample reconstitution. The method was validated for the analysis of PPI-2458 in 200 µl human plasma samples over a concentration range from 0.0200 ng/ml to 10.0 ng/ml in 200 µl sample volumes, and from 5.00 ng/ml to 2500 ng/ml in 100 µl sample volumes. Aliquots of study samples were transferred to 2 ml 96 well deep well plates. To these were added 50 µl of PPI-2458-d8 internal standard at 2 ng/ml and 100 ng/ml for low-range and high-range samples, respectively. Samples were then treated with 50 µl 95% H2O/CH3CN and briefly vortexed. Samples were then precipitated with 400 µl (high range) or 800 µl (low range) CH3CN, and centrifuged at 3250 rpm for 10 minutes. Aliquots of supernatant (400 µl, high range; 800 µl, low range) were then evaporated to dryness under nitrogen at 55°C in a TurboVap concentrator (Biotage, Uppsala, Sweden). The dried residues were then reconstituted with 100 ml of 90:10 H2O/CH3CN. Quantitation was performed using LC conditions as follows: column, 2.0 × 20 mm Phenomenex Mercury MS Luna C8 (Phenomenex, Torrence, CA); mobile phases: A = 85:5:10 H2O/CH3CN/25 mM NH4OAc (aq, pH 3.5), B = 90:10 CH3CN/25 mM NH4OAc (aq, pH 3.5); flow rate 700 µl/min; injection volume: 30 µl (low range), 5 µl (high range); gradient 20% B for 0.5 minutes, then 20% → 60% B in 1.1 minutes, then 60% → 90% B in 0.1 minute. Chromatograms were acquired on an Applied Biosystems API4000 triple quadrupole mass spectrometer operating in ESI+ mode with MRM Q1 and Q3 transition mass (amu) as follows: PPI-2458, 442.4 and 375.2; PPI-2458-d8 (internal standard), 450.4 and 383.3; +16 metabolite, 458.4 and 391.2; and +32 metabolite, 474.4 and 313.2. Chromatograms were integrated using Applied Biosystems Analyst software, and plasma concentrations of the metabolites were determined from peak areas and averaged response factors of the appropriate calibration standards.

Pharmacodynamic analyses were performed on white blood cell lysates obtained before the first dose then at 4 hours, 24 hours, and 48 hours (before the second dose); and on day 15 before the first dose, and 4 hours postdose for patient 1. For patient 2, the day-15 samples were not obtained due to withdrawal from the study. Blood samples of 4 ml were collected into 4 ml Sodium Citrate Vacutainer Tubes (362760; Becton-Dickinson, Franklin Lakes, NJ) and mixed gently by inversion. They were then centrifuged at room temperature in a swinging bucket rotor for 30–45 minutes at 1700g to separate red blood cells. Mononuclear cells and platelets were then resuspended into plasma and decanted. The cell suspension was then diluted to 11 ml with cold PBS and divided into two 5 ml aliquots. Each tube was further diluted with 10 ml of cold PBS and centrifuged at 500g in a swinging bucket rotor for 10 minutes at 4°C. The duplicate cell pellets were then frozen and stored at \(-80\)°C and shipped to TGA Sciences (Medford, MA) for further processing. Free MetAP2 levels were determined by ELISA as described earlier (Bernier et al., 2004). The total protein content in the lysates was determined using the bicinchoninic acid (BCA) method (Stoscheck, 1990).

#### Results

### Identification of Metabolites

PPI-2458 was incubated with microsomes from five species (mouse, rat, dog, monkey, and human), and the products were analyzed by LC-MS. Four prominent metabolite peaks were generated by all species evaluated. All four of these metabolites (M1, RT = 26.7 minutes; M2, RT = 20.8 minutes; M3, RT = 20.1 minutes; M4, RT = 17.6 minutes) had molecular ion masses of 441, corresponding to an addition of 16 amu and consistent with the addition of one oxygen atom into the molecular structure of PPI-2458 (Supplemental Fig. 2). The intensity of these metabolite peaks correlated with the CYP3A activity of the microsomes as obtained from the supplier (i.e., mouse and monkey > human and dog). Likewise, microsomal metabolism of PPI-2458 was efficiently blocked by KTZ, a potent inhibitor of CYP3A, with IC50(KTZ) \(-100\) nM. Additional metabolite peaks of lower intensity and earlier retention times were also observed in some of the samples. These peaks had molecular ions corresponding to...
addition of 32 (M5, M6; incorporation of two oxygen atoms) or 2 amu (incorporation of one oxygen [+16] and demethylation [−14]) relative to PPI-2458. Incubations with rat S9 revealed the formation of a glutathione conjugate of PPI-2458 (RT 20.8 minutes, m/z = 732) in addition to M1–M4. The formation of this conjugate was independent of NADPH, and its mass was consistent with direct addition to the parent compound.

Analysis of product ion spectra of the prominent +16 amu metabolites in comparison with that of PPI-2458 provided little structural information due to the complex fragmentation patterns of these molecules. It was possible to determine that M1, with a retention time of 26.7 minutes, had undergone modification on the valine moiety of PPI-2458. It was also apparent that the other +16 metabolites were unmodified on valine. The +16 metabolites with retention times of 20.1 and 20.8 minutes, in particular, had nearly identical fragmentation patterns, suggesting that these two metabolites might be stereoisomers. To establish the detailed structures of M1–M6, representing the most prominent metabolites, microsomal preparations were generated at a large scale, and samples of each metabolite were isolated in sufficient quantity for NMR (100 μg in low volume, susceptibility-matched NMR tubes). The structures deduced by NMR were then confirmed by chemical synthesis and are shown in Fig. 2.

**M1.** In agreement with the MS data, the NMR of M1 showed a loss of the βCH D-Val resonance (H19) at 2.04 ppm in PPI-2458. In addition, the αCH and γCH3 resonances (H20 and Me18) shifted downfield and collapsed to singlets. The data are thus consistent with β-hydroxylation of the PPI-2458 valine moiety. Chemical synthesis of M1 was accomplished by acylation of the N-hydroxysuccinimidy carbonate ester of fumagillol with H-D-β-hydroxovaline amide, which was prepared in five steps from methyl (S)-(−)-3-(tert-butoxycarbonyl)-2,2-dimethyl-4-oxazolidinecarboxylate (Supplemental Fig. 1).

**M2 and M3.** Metabolites M2 and M3 eluted with very similar retention times by LC (Supplemental Fig. 2), and their NMR spectra were also found to resemble each other. The distinguishing features of each were the disappearance of the olefinic proton H3 at 5.24 ppm, the appearance of new signals near 3 ppm, and the upfield shift of the prenyl methyl groups (Me1 and Me2) to 1.3 ppm. These data are consistent with nonstereospecific epoxidation of the side chain double bond, rather than hydroxylation at a single carbon. The NMR spectra of M2 and M3 differ only slightly, with the most noticeable dissimilarities in the region between 1.5 and 3 ppm containing H3, 4, 5, and 6. H3 and H6 could be distinguished from each other by rotating frame Overhauser effects between H3 and H2, and between H6 and H11.

M2 and M3 could be synthesized simultaneously by treatment of PPI-2458 with dimethyldioxirane, generated in situ from potassium peroxomonosulfate and acetone, followed by resolution of the diastereomers by preparative HPLC. A tentative stereospecific assignment of M2 and M3 was achieved by oxidation of PPI-2458 with Shi’s fructose-based catalyst, 1,2:4,5-di-O-isopropylidene-β-D-erythro-2,3-hexodiulo-2,6-pyranose (Wang et al., 1997). A single species was produced, which coeluted with M2. In analogy with the determinations published by Wang and coworkers, the structures of M2 and M3 were assigned to the R and S configurations at C3, respectively, as shown in Fig. 2.

**M4.** The NMR spectrum of M4 shows a disappearance of a methyl singlet corresponding to Me2 in the spectrum of PPI-2458, and the
appearance of a two-proton singlet at 3.9 ppm. The resonance of H3 also shifts downfield by over 0.2 ppm. These data suggest hydroxylation of C2 to form M4.

Chemical synthesis of M4 was accomplished by oxidation of PPI-2458 with SeO3, which is known to yield the trans-allylic alcohol when applied to prenyl groups (Bhalerao and Rapoport, 1971). The NMR of the synthetic product lacked two small signals seen in the authentic metabolite at 1.9 and 4.1 ppm, although the major signals were in good alignment (Supplemental Fig. 3). This suggests that M4 consists predominantly of the cis alcohol, with a minor component of the trans alcohol, in an approximate ratio of 5 to 1 (Fig. 2).

**M5 and M6.** MS studies indicated that M5 and M6 were doubly oxidized (+32) with one oxygen on the D-Val. NMR spectra of the synthetic product lacked two small signals seen in the authentic metabolite at 1.9 and 4.1 ppm, although the major signals were in good alignment (Supplemental Fig. 3). This suggests that M4 was primarily responsible for the metabolism of PPI-2458.

**Initial rate kinetics for M1–M6.** Table 2 shows the average linear rate of formation of the metabolites when applied to prenyl groups (Bhalerao and Rapoport, 1971). The average rates across all the metabolites, however, were comparable: 0.12 μmol/min/pmol CYP3A4 and 0.094 μmol/min/pmol CYP3A5.

**PPI-2458 Inhibition of P450 Activity.** Concentrations of PPI-2458 up to 40 μM failed to inhibit CYP3A4 mediated oxidation of dibenzofluorescene, whereas KTZ inhibited this reaction with IC50 = 9.8 nM (data not shown). To explore the potential for inhibition of other CYP3A substrates, PPI-2458 was incubated with midazolam, nifedipine, or testosterone in pooled human liver microsomes and compared with inhibition by KTZ. Initial rate conditions, linear with respect to time and microsomal protein concentration, were established, and then inhibition was assessed in the presence of 25–400 μM PPI-2458 or 0.02–0.5 μM ketoconazole. Lineweaver-Burk and Dixon analysis indicated that inhibition by PPI-2458 was consistent with competitive mechanism. Data were fit to the Michaelis-Menten model of competitive inhibition as well as to several models of atypical kinetics. The resulting kinetic parameters are shown in Table 3. The Michaelis-Menten model was the best fit for all but KTZ inhibition of nifedipine oxidation, which displayed negative cooperativity. KTZ inhibited the CYP3A-mediated formation of 1’-hydroxyximadazolam, oxidized nifedipine, and 6β-hydroxytestosterone with K_i values of 0.007, 0.011, and 0.049 μM, respectively. The corresponding K_i values exhibited by PPI-2458 were 58, 403, and 287 μM.

**In Vitro Inhibition of MetAP2 Activity and HUVEC Proliferation.** We have reported an assay that is suitable for analysis of slow, tight-binding fumagillin-based MetAP-2 inhibitors (Arico-Muendel et al., 2009). Thus, this assay records the fraction of active enzyme remaining during an 8-hour incubation with inhibitor. The activities of M1–M6 in this assay are shown in Table 4. PPI-2458 inhibited MetAP2 to approximately 12% of enzyme-only control in 1 hour, and to 3% at 8 hours. The six metabolites, M1 demonstrated a similar level of activity. M2 and M4 showed slower initial activity but reached ~90% inhibition at the
end of the experiment, whereas M3 was only ~40% inhibited at this time. The potencies of M5 and M6 roughly tracked those of M2 and M3, respectively. A non-metabolite analog of M4, PPI-4338, was nearly inactive.

Inhibition of HUVEC Proliferation. HUVEC cultures were incubated with purified metabolites for 3 days, and the percentage of cell growth was determined with methylthiazolyldiphenyl-tetrazolium bromide (thiazolyl blue tetrazolium bromide) relative to vehicle control. A representative HUVEC assay is shown in Fig. 3, and all the assay data are summarized in Table 4. Under these conditions, M1 exhibited the highest potency, close to that of PPI-2458 (EC50 = 0.3 nM versus 0.1 nM). The activity of M2 was reduced ~10-fold to 1.3 nM, whereas the remaining metabolites inhibited near 10 nM.

Metabolite PD and PK Studies

MetAP2 Inhibition after Single-Dose Administration of PPI-2458 in Rats. An ELISA-based assay was employed to determine the free MetAP2 levels in rat blood and tissue samples after treatment with PPI-2458 and its synthesized metabolites. Briefly, cells were lysed and treated with a PPI-2458–biotin conjugate, which enabled capture of free MetAP2 onto immobilized streptavidin and detection/quantitation by an anti-MetAP/horseradish peroxidase dual antibody system (Bernier et al., 2004). Compounds were administered as single doses, either intravenously or orally, at 0.3 and 3.0 mg/kg. To follow the earlier research suggesting the utility of PPI-2458 for treatment of non-Hodgkin’s lymphoma (Cooper et al., 2006), samples were collected from blood, lungs, lymph nodes, and thymus 24 hours after administration. Free MetAP2 levels, as a percentage of the vehicle, are shown in Fig. 4. After intravenous administration, similar profiles for average free MetAP2 levels in different compartments were observed across all the compounds tested; however, individual measurements except for blood were also highly variable. In contrast, PPI-2458 and the metabolites exhibited more differentiated activity after oral administration. PPI-2458 and M1 showed similar levels of inactivation, followed by somewhat less effective M2, M3, and M4. Thus, significant inhibition by a 0.3 mg/kg oral dose was only observed for PPI-2458 and M1, whereas all compounds were effective at 3 mg/kg. Effectiveness as a function of compartment followed the order blood > liver > lungs > lymph nodes > thymus.

Plasma samples were also obtained for all studies at 4 hours and 24 hours after the dose to assess exposure. For the intravenous arm, levels of the parent compound and metabolites were below the level of quantitation for all compounds at both time points, and only trace quantities (~0.3 ng/ml) could be detected at the lower oral dose (unpublished data). For animals dosed at 3.0 mg/kg orally, quantifiable levels of each analyte could be observed at 4 hours for the 3 mg/kg dose, but only for M1 at 24 hours (Table 5). M2, M3, and M4 could also be detected after a 3 mg/kg oral dose of PPI-2458.

PK/PD Studies in Humans. As part of a phase 1 dose escalation study, PPI-2458 was administered to two patients with non-Hodgkin lymphoma. Oral doses of PPI-2458 at 15 mg/kg were given every other day, and plasma samples for PK/PD analysis were collected at 15 minutes, 30 minutes, and 1, 2, 3, 4, 6, 8, 24, and 48 hours after the first dose, as well as before the dose and 4 hours after the dose on day 15 (one patient). For both subjects, the uninhibited (free) MetAP2 levels in white blood cell samples were below the quantitation limit 4 hours after the dose (Table 6), indicating rapid target engagement and inactivation after oral dosing. As a percentage of predose free MetAP2 level, this corresponded to <1.5% and <6.0% for patients 1 and 2, respectively, due to the 4-fold difference in predose free MetAP2 (Table 6). For both subjects, inhibition remained high (1.6% and <6.0% free MetAP2, respectively) for 24 hours, and showed partial recovery after 48 hours (6.6 and 17.1% free MetAP2, respectively).

Levels of PPI-2458 and M1–M6 were measured to determine PK parameters (Table 7). The exposure profiles for both patients are shown in Fig. 5. PPI-2458 accounted for approximately 10% of the total measured exposure, with maximum plasma concentrations of 77.7 and 32.3 ng/ml attained within an hour of initial administration for both subjects. Elimination of PPI-2458 was monophasic, with t1/2 = 2.1 and 1.1 hours. Of the species monitored in the study, the metabolites accounted collectively for nearly 90% of the exposure, with the greatest contribution by M2 (~30%), followed by M3 (~20%). On the other hand, the exposure due to M1 is almost negligible. Average Tmax (time of maximum plasma concentration) values within an hour were also observed for M1–M4, suggesting rapid formation of these species, whereas those for M5 and M6 were slightly longer. An apparent elimination phase followed from the time of the peak metabolite concentrations. Metabolites M2, M3, and M4 had apparent elimination half-lives of approximately 1 hour, whereas M5 and M6 tended to have longer half-lives (closer to 2 hours) during this phase.

For most of the metabolites, a second elimination phase, where metabolite concentrations initially increased and then either stabilized or diminished at a slower apparent elimination rate, was seen at 4 hours after the dose. The slight increases or maintenance of metabolite concentrations occurred from 6 hours to 24 hours after the dose for patient 1 and from 4 hours to 8 hours after the dose for patient 2. During this second phase, concentrations of M5 and M6 rose to levels similar in magnitude to those of M2 and M3. M4 showed a trend similar to that of M5 and M6 with lower nominal concentration levels.

<p>| TABLE 3 |
| Kinetics of inhibition of microsomal metabolism of representative drugs |
| Results are expressed as estimate (± S.E.). |</p>
<table>
<thead>
<tr>
<th>CYP3A Substrate</th>
<th>Modifier</th>
<th>Kᵢ (µM)</th>
<th>Kᵢa (µM)</th>
<th>Vmax (nmol min⁻¹ m₄HLM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midazolam</td>
<td>KTZ</td>
<td>0.007</td>
<td>1.7 (0.6)</td>
<td>2.6 (0.3)</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>PPI-2458</td>
<td>0.011</td>
<td>1.9 (0.3)</td>
<td>2.6 (0.1)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>KTZ</td>
<td>0.049</td>
<td>145 (48)</td>
<td>5.8 (1.4)</td>
</tr>
</tbody>
</table>

Discussion

This study of the metabolism of PPI-2458 was driven by observations of efficacy in animal models for cancer (Cooper et al., 2006; Hannig et al., 2006) and rheumatoid arthritis (Hannig et al., 2007; Lazarus et al., 2008) after oral dosing, despite apparent low oral bioavailability of the parent compound. As a first step toward characterization of PPI-2458 metabolism in vivo, we studied the effects of treatment with liver microsomal preparations. Six major species were identified, corresponding to four single and two double oxidation products, as well additional minor species and a glutathione adduct formed in rat S9 preparations. Identification of CYP3A4/5 as playing a key role in the formation of M1–M6 was suggested by the effectiveness of KTZ in suppressing metabolism, and was demonstrated explicitly by phenotyping experiments that showed a clear correlation between the activity of these CYP isoforms and metabolite formation. In addition, measurement of kinetic parameters using recombinant CYP3A4 and CYP3A5 indicated similar intrinsic clearance rates (Vmax/Km) for both enzymes. The proportions of the
### TABLE 4
Inhibition of human MetAP2 and HUVEC proliferation by metabolites and PPI-4338

<table>
<thead>
<tr>
<th>Compound</th>
<th>MetAP2 (% activity)</th>
<th>HUVEC EC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPI-2458</td>
<td>2.8</td>
<td>0.095</td>
</tr>
<tr>
<td>M1</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>M2</td>
<td>10</td>
<td>1.3</td>
</tr>
<tr>
<td>M3</td>
<td>58</td>
<td>6.5</td>
</tr>
<tr>
<td>M4</td>
<td>9</td>
<td>7.1</td>
</tr>
<tr>
<td>M5</td>
<td>6</td>
<td>8.8</td>
</tr>
<tr>
<td>M6</td>
<td>39</td>
<td>11.7</td>
</tr>
<tr>
<td>PPI-4338</td>
<td>90</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a Quantitated as percentage activity remaining after 8-hour exposure to compound.*
metabolites formed in microsomes generally parallel the kinetics observed from recombinant CYP3A4/5, where the intrinsic clearance of M1 lags that of M2–M4. Relative to KTZ, PPI-2458 was found to minimally block CYP metabolism of three representative drugs, with $K_i$ in the high micromolar range.

Through a large-scale preparation, sufficient quantities of M1–M6 were isolated and purified to enable two-dimensional NMR studies. As a result, detailed structures for each metabolite could be proposed, which were confirmed by independent chemical synthesis. The structures for M1–M6 are consistent with P450-mediated oxidation reactions involving attack at specific sites on hydrophobic groups at the periphery of the molecule. Of the few examples of published fumagillin metabolites, it is noteworthy that oxidation of fumagillin with m-chloroperbenzoic acid yields a mixture of diastereomeric triepoxides corresponding to M2 and M3 in PPI-2458 (Halász et al., 2000). The same study reported these species as minor impurities in commercial fumagillin resulting from degradation over time.

Metabolism of fumagillin derivatives has been studied in the case of TNP-470 (Placidi et al., 1995) and CKD-732 (Myung et al., 2002). In vivo, TNP-470 is rapidly degraded to an unsubstituted carbamate by esterases, which in turn is processed by microsomal epoxide hydrolase into an inactive bicyclic structure. For CKD-732, the polyolefinic side chain of this molecule is replaced a cinnamyl ester functionalized with a 4-dimethylaminoethoxy group, whereas the core and the isobutenyl containing C4 substituent are preserved. Microsomal metabolism of CKD-732 yielded a single predominant species, identified as the N-oxide of the dimethylamino moiety, and 13 other species of much lesser abundance that were characterized by LC-MS. Several of these were assigned to oxidations of one of the C4 terminal methyl groups. However, double bond epoxidation, as was observed with M2 and M3 in the case of PPI-2458, would also be consistent with the limited MS fragmentation data.

An additional modification proposed in several of the metabolites involved opening of the parent epoxide groups by addition of water to
form a fused bicyclic structure as in the case of TNP-470. Although we did not observe significant formation of such water adducts in this study, we did find them as significant products of PPI-2458 degradation in acidic conditions (Arico-Muendel et al., unpublished data).

Testing the isolated compounds M1–M6 in hMetAP2 and HUVEC assays demonstrated that the inhibitory activity of PPI-2458 was preserved in all the major metabolites. The good correlation of the biochemical and cellular assays establishes a link between compound-mediated enzyme inhibition and antiproliferative activity in a cell type relevant to angiogenesis. The resulting structure–activity relationships may be considered in light of the published X-ray structure of MetAP2–fumagillin (Liu et al., 1998). The C4 side chain was found to be relevant to angiogenesis. The resulting structure

### TABLE 5

<table>
<thead>
<tr>
<th>PPI-2458 ng/ml</th>
<th>M1 ng/ml</th>
<th>M2 ng/ml</th>
<th>M3 ng/ml</th>
<th>M4 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h 24 h</td>
<td>4 h 24 h</td>
<td>4 h 24 h</td>
<td>4 h 24 h</td>
<td>4 h 24 h</td>
</tr>
<tr>
<td>0.25 BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>1.7 BLQ</td>
<td>0.6 BLQ</td>
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<tr>
<td>0.59 BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>3.8 BLQ</td>
<td>1.4 BLQ</td>
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<tr>
<td>0.82 BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>2.5 BLQ</td>
<td>0.94 BLQ</td>
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</table>

### TABLE 6

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Day</th>
<th>WBC Count*</th>
<th>PD Time Point</th>
<th>Total Protein</th>
<th>Free MetAP2</th>
<th>Predose Free MetAP2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(10^3/μl)</td>
<td></td>
<td>mg/ml</td>
<td>mg/ml</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>6.8</td>
<td>Predose</td>
<td>1.82</td>
<td>61.8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 h</td>
<td>1.60</td>
<td>BLQ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>2</td>
<td>7.6</td>
<td>24 h</td>
<td>1.48</td>
<td>0.985</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>48 h (before dose 2)</td>
<td>1.37</td>
<td>4.06</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>7.3</td>
<td>Before day 15 dose</td>
<td>1.20</td>
<td>6.48</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 h after day 15 dose</td>
<td>2.47</td>
<td>BLQ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;1.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.9</td>
<td>Predose</td>
<td>0.861</td>
<td>15.6</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 h</td>
<td>1.03</td>
<td>BLQ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;6.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.1</td>
<td>24 h</td>
<td>0.481</td>
<td>2.56</td>
<td>17.1</td>
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<tr>
<td></td>
<td>ND</td>
<td>48 h (before dose 2)</td>
<td>0.786</td>
<td>2.66</td>
<td>17.1</td>
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</table>

<sup>a</sup> WBC, white blood cell.

<sup>b</sup> Clinical hematology panel results from samples independent of those drawn for pharmacodynamic analysis.

<sup>*</sup> BLQ determined to be <0.940 ng/ml from LLOQ multiplied by the lowest sample dilution factor used in the assay (0.188 ng/ml × 5).
TABLE 7
Pharmacokinetic parameters for PPI-2458 and its metabolites (individual and aggregated) in two non-Hodgkin lymphoma patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patient</th>
<th>PPI-2458</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>Summed Analytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.7</td>
<td>0.7</td>
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NA, not available;

$^a$ For metabolites, an apparent half-life reflecting the rate of elimination during the first phase, where plasma concentrations go from peak levels to levels near the lower limit of quantitation, is displayed.

$^b$ $CL/F$ (initial dose bioavailability adjusted clearance) and $V_{ss}/F$ (steady-state volume of distribution) are based on the dose amount administered and are therefore computed only for PPI-2458 and the summed analytes.

Fig. 5. Day 1 pharmacokinetic profile of PPI-2458 and its metabolites after a 15 mg oral dose of PPI-2458 in patient 1 (top) and patient 2 (bottom). Points BLQ are arbitrarily set to a concentration of 0.05 ng/ml.
MetAP2 in blood, with <20% free enzyme measured at 48 hours after dosing. Analysis of plasma samples indicated that most of the exposure was due to metabolites, predominantly M2 and M3. The exposure profiles indicate that PPI-2458 is rapidly metabolized favoring M2 and M3 initially. That M1 levels are nearly negligible, whereas the secondary metabolites M5 and M6 appear rapidly, suggests that D-Val hydroxylation is inefficient relative to epoxidation, and that M5 and M6 form primarily via M2 and M3. Because M2 and M3 are somewhat less effective inhibitors of MetAP2, development of second-generation compounds could focus on suppressing this metabolic pathway. Overall, the results accord with the trend observed in primate studies (not shown) that indicate an increasing level of exposure due to metabolites in higher species.

In conclusion, this work has identified the CYP-generated metabolites of PPI-2458 and determined their activity in vitro. The metabolites are shown to contribute significantly to pharmacologically active compound exposure in vivo. In humans, the initial results of a phase 1 study in patients has demonstrated effective inhibition of MetAP2 administered orally.

Acknowledgments

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Authorship Contributions

Participated in research design: Arico-Muendel, Benjamin, Blanchette, Morgan, Thompson, Wakefield, Westlin.


Contributed new reagents or analytic tools: Centrella, Labenski.


Wrote or contributed to the writing of manuscript: Arico-Muendel, Ciazzo, Gradhand, Wakefield, Westlin.

References


Ashraf S, Mapp PL, and Walsh DA (2010) Angiogenesis and the persistence of inflammation in active compound exposure in vivo. In humans, the initial results of studies (not shown) that indicate an increasing level of exposure due to what less effective inhibitors of MetAP2, development of second-generation compounds could focus on suppressing this metabolic pathway. Overall, the results accord with the trend observed in primate studies (not shown) that indicate an increasing level of exposure due to metabolites in higher species.

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